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Modulation of Multidrug Resistance-Associated Protein 2 (Mrp2) and Mrp3 Expression and Function with Small Interfering RNA in Sandwich-Cultured Rat Hepatocytes

Xianbin Tian, Maciej J. Zamek-Gliszczynski, Peijin Zhang,[1] and Kim L. R. Brouwer

Division of Drug Delivery and Disposition, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina

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ABSTRACT

Canalicular multidrug resistance-associated protein 2 (Mrp2) and basolateral Mrp3 mediate the excretion of organic anions, including conjugated and unconjugated xenobiotics and bile acids, from the liver. The utility of RNA interference to specifically knock down the expression and function of transport proteins was demonstrated in sandwich-cultured rat hepatocytes, which exhibit functional and properly localized Mrp2 and Mrp3 over time in culture. Specific knockdown of Mrp2 (~50% decrease in expression) resulted in an ~45% decrease in the biliary excretion index of carboxydichlorofluorescein (CDF) (9.3% versus 16.5%), but did not affect Mrp3 or radixin expression. Specific Mrp3 knockdown (~50% decrease in expression) resulted in significantly higher accumulation of CDF in cells + bile canaliculi (32.3 ± 2.5 versus 24.4 ± 4.3 pmol/mg of protein/10 min), but no change in cellular accumulation (13.7 ± 2.2 versus 15.6 ± 4.0 pmol/mg of protein/10 min), consistent with an ~60% increase in the biliary excretion index of carboxydichlorofluorescein. The extent of protein knockdown was in good agreement with changes in carboxydichlorofluorescein disposition. In conclusion, modulation of drug transporters in sandwich-cultured rat hepatocytes by small interfering RNA treatment is a feasible in vitro approach to study the expression and function of drug transport proteins.

The multidrug resistance-associated proteins (MRPs) are a subfamily of the ATP-binding cassette transport protein family involved in drug resistance and excretion of organic anions (Schinkel and Jonker, 2003). At least nine members of this subfamily have been characterized; MRP2 and MRP3 are two key members of this group expressed in the liver. MRP2, located on the canalicular membrane of hepatocytes, and MRP3, a basolateral membrane protein, function as efflux transporters for organic anions (e.g., drugs, bile acids, and their phase II anionic conjugates) and play important roles in resistance to chemotherapy in human carcinoma (Kruh and Belinsky, 2003). Mutations in human MRP2 are associated with Dubin-Johnson syndrome, an autosomal recessive disorder resulting in chronic conjugated hyperbilirubinemia (Keitel et al., 2000). Expression of MRP2 and MRP3 in carcinoma cells resulted in drug resistance during chemotherapy treatment (Norris et al., 1996; Nies et al., 2001; Young et al., 2001). Modulation of MRP2 and/or MRP3 may, therefore, have valuable therapeutic implications. Mrp2 and Mrp3 are the rodent isoforms of human MRP2 and MRP3. Expression, localization, and function of Mrp2 and Mrp3 in biliary and basolateral excretion, respectively, of organic anions have been studied extensively in vitro and in vivo (Hirohashi et al., 1999; Kool et al., 1999; Mottino et al., 2000). Mutations in Mrp2 also cause hyperbilirubinemia in rodents (Buchler et al., 1996).

Primary rat hepatocytes cultured in a sandwich configuration (between two layers of gelled collagen) form extensive canalicular networks (LeCluyse et al., 1994). Previous studies have shown that canalicular and basolateral efflux transport proteins involved in drug transport (e.g., P-glycoprotein, Mrp2, and Mrp3) are expressed and functional when rat hepatocytes are cultured in a sandwich configuration; proteins are localized to the appropriate plasma membrane domain over time in culture (Liu et al., 1999b; Annaert et al., 2001; Zhang et al., 2001; Luttringer et al., 2002). The functional activity of Mrp2 and Mrp3 can be demonstrated with fluorescent 5 (and 6)-carboxy-2′,7′-dichlorofluorescein (CDF),

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[1] Current address: Qualyst, Inc., Research Triangle Park, NC.

ABBREVIATIONS: Mrp, multidrug resistance-associated protein; CDF, 5 (and 6)-carboxy-2′,7′-dichlorofluorescein; siRNA, small interfering RNA; HepG2, human hepatoblastoma; siFL, siRNA sequences targeting firefly luciferase; siMrp2 or siMrp3, siRNA sequences targeting Mrp2 or Mrp3; HBSS, Hanks' balanced salt solution; GF120918, *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; ANOVA, analysis of variance.

a substrate of both Mrp2 and Mrp3. Nonfluorescent CDF diacetate passively diffuses into hepatocytes, where it is rapidly hydrolyzed by intracellular esterases to fluorescent CDF, which is actively excreted by Mrp2 across the canalicular membrane into the bile canalicular networks of sandwich-cultured rat hepatocytes, or undergoes basolateral excretion into cell medium by Mrp3 (Zamek-Gliszczynski et al., 2003). Well preserved liver-specific functions in sandwich-cultured rat hepatocytes make this a valuable in vitro model to study the expression and function of transport proteins, and predict in vivo hepatobiliary clearance (Liu et al., 1999a).

RNA interference involves the use of double-stranded RNA to mediate sequence-specific suppression of gene expression. In brief, double-stranded small interfering RNA (siRNA), typically 22 to 23 nucleotides in length, is created by using the unique sequence of a target gene as a template, and is then introduced into host cells. The endogenous RNA-induced silencing complex is activated by the siRNA and guided to the mRNA of the target gene. The mRNA of the target gene subsequently is cleaved into small fragments; thus, expression of the target gene is suppressed before translation can occur (Hannon, 2002; Dykxhoorn et al., 2003). Recent progress has been made in the application of siRNA to specifically knock down gene expression in cell lines and, to a more limited extent, in primary mammalian cells and in vivo (Krichevsky and Kosik, 2002; Cioca et al., 2003; Konishi et al., 2003; Nieth et al., 2003; Reid et al., 2003; Wu et al., 2003). However, to date, siRNA has not been used in primary hepatocytes to specifically knock down expression of important drug transport genes. The objective of this study was to evaluate the utility of targeted knockdown of transport protein in the study of transporter function in primary rat hepatocytes.

Materials and Methods

Cell Culture of Human Hepatoblastoma (HepG2) Cells and Primary Rat Hepatocytes. HepG2 cells were maintained in Dulbecco's modified Eagle's medium/F-12 plus 10% fetal bovine serum. Rat hepatocytes were isolated from male Wistar rats (220–300 g) by in situ collagenase perfusion (Annaert et al., 2001). Cells were seeded at a density of 1.5×10^6 cells per well onto polystyrene six-well plates precoated with rat-tail type I collagen and were overlaid with gelled collagen 24 h after plating. Rat hepatocyte cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 0.1 μM dexamethasone and 1% ITS culture supplement (6.25 mg/ml insulin, 6.25 mg/ml transferrin, 6.25 μg/ml selenous acid, 5.35 mg/ml linoleic acid, and 1.25 g/ml bovine serum albumin). Medium was changed every 24 h.

Synthesis of siRNA. siRNA sequences targeting rat Mrp2 and Mrp3 were chosen with RNAi OligoRetriever (http://www.cshl.org/public/science/hannon.html) for the T7 RNA polymerase protocol. Single-stranded RNA was synthesized with a T7-MEGAshortscript high yield transcription kit (Ambion, Austin, TX) according to the manufacturer's instructions. To make double-stranded siRNA, sense and antisense of single-stranded RNAs in equimolar amounts were denatured and annealed with a thermocycler (95°C, 5 min; 70°C, 5 min; 50°C, 5 min; 25°C, 5 min). The integrity of siRNA was examined with a 2% agarose gel. The following three siRNA antisense sequences targeting Mrp2 were synthesized: siMrp2AB1, GGCUAUAUCUGUGCAAUCCUA (UA 3′ overhang in antisense strand and AA 3′ overhang in sense strand); siMrp2AB2, GGCUAGGAAGCAGUACACCAU (AU 3′ overhang in antisense strand and AA 3′ overhang in sense strand); and siMrp2AB3, GGCAGUAGGGUGGUGGUCCAU (AU 3′ overhang in antisense strand and UG 3′ overhang in sense strand). The following antisense sequence was used to target Mrp3: GGUCCAAGGACCUGCCUCCCA (CA 3′ overhang in antisense strand, AG 3′ overhang in sense strand). The siRNA sequence [antisense, GUGCGCUGCUGGUGCCAACUU (UU 3′ overhangs in both strands)] targeting firefly (*Photinus pyralis*) luciferase (siFL) was adapted from a known potent sequence for this protocol (Miyagishi and Taira, 2002).

Intracellular Tracking of siRNA. siRNA was attached covalently to the Cy3 dye in a one-step chemical reaction with the LabelIT siRNA Tracker intracellular localization kit (Mirus Corporation, Madison, WI). Ten micrograms of siRNA was incubated with 10 μl of reconstituted Cy3 labeling reagent at 37°C for 1 h. Labeled siRNA was purified by a regular ethanol precipitation method to remove excess Cy3. Cy3-labeled siRNA was transferred into sandwich-cultured rat hepatocytes as described in the following section ("siRNA Transfection"). Cell images were taken with an Axiovert 100TV inverted microscope (Carl Zeiss Inc., Thornwood, NY).

siRNA Transfection. HepG2 cells were seeded at a density of 2×10^5 cells per well onto polystyrene 12-well plates 24 h before transfection. A mixture of 100 ng of firefly luciferase expression plasmid (pGL3-FL) and 100 ng of *Renilla reniformis* luciferase expression plasmid (pRL-TK) and various amounts of firefly luciferase siRNA were used per well. Transfections of sandwich-cultured rat hepatocytes were performed at 20 h after plating, unless specifically indicated. Two micrograms of siRNA was used per well in six-well plates. siRNA and DNA were formulated for transfection using a TransMessenger transfection reagent kit (QIAGEN, Valencia, CA). In brief, siRNA was condensed in Enhancer EC at a ratio of 1:2 (micrograms of total RNA and/or DNA/microliter of Enhancer) and formulated with TransMessenger at a ratio of 1:4 (micrograms of RNA and/or DNA/microliter of TransMessenger) and then incubated with cells. For transfections of HepG2 cells, medium was changed after an overnight incubation with siRNA. For transfections of sandwich-cultured rat hepatocytes, cells were incubated with siRNA for 4 h and then were overlaid with collagen. The effect of transfection was examined 48 h later.

Luciferase Activity Assay. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). In brief, HepG2 cells were lysed in 100 μl of passive lysis buffer. Cell lysate (20 μl) was mixed with 100 μl of Luciferase Assay Reagent II to measure firefly luciferase activity. One hundred microliters of Stop and Glo reagent was then added and mixed to measure the *R. reniformis* luciferase activity. Luminescence intensity was measured with a Monolight 3010 Luminometer (BD Biosciences, San Jose, CA).

Immunoblot Analysis. Cells were harvested 48 h post-transfection and lysed in 1% SDS, 1 mM EDTA plus Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The protein concentration of the clear cell lysate was determined with the BCA protein assay reagent kit (Pierce Biotechnology, Inc., Rockford, IL). Fifty micrograms of total protein per lane was resolved by electrophoresis on NuPAGE 4 to 12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and were transferred onto polyvinylidene difluoride membranes. The following antibodies were used to probe the membranes: anti-Mrp2 (M_2III-6; Alexis Biochemicals, San Diego, CA), anti-Mrp3 (kind gift from Dr. Yuichi Sugiyama, University of Tokyo, Tokyo, Japan), anti-radixin (Chemicon International, Temecula, CA), and anti-β-actin (MAB1501; Chemicon).

Assessment of Mrp2 and Mrp3 Function with CDF Disposition. Cells were rinsed twice with Hanks' balanced salt solution (HBSS; 2 ml, 37°C), incubated (10 min, 1.5 ml, 37°C) with 2 μM CDF diacetate (Molecular Probes, Eugene, OR), and rinsed twice with cold HBSS (2 ml, 4°C). Cell images were taken with an Axiovert 100TV inverted microscope after a washout period of ~10 min. Accumulation of CDF in cells + bile canaliculi and cellular accumulation were quantified in sandwich-cultured rat hepatocytes preincubated (10 min, 2 ml, 37°C) with standard HBSS or Ca^{2+}-free HBSS, respectively (Liu et al., 1999c).

Thereafter, sandwich-cultured rat hepatocytes were incubated with 2 μM CDF diacetate (10 min, 2 ml, 37°C). In siMrp2-treated sandwich-cultured rat hepatocytes, cells were rinsed four times with cold HBSS (2 ml, 4°C) and lysed (1 ml, 0.5% Triton X-100 in phosphate-buffered saline). In siMrp3-treated sandwich-cultured rat hepatocytes, after incubation with CDF diacetate, cells were rinsed four times with warm HBSS (2 ml, 37°C) and were incubated with HBSS (30 min, HBSS changed every 10 min; 2 ml, 37°C) to allow time for intracellular CDF to undergo appreciable excretion before rinsing the sandwich-cultured rat hepatocytes four times with cold HBSS and lysing the cells (as above). CDF fluorescence in lysate was quantified with fluorescence spectrophotometry (λ_{ex} = 485 nm, λ_{em} = 590 nm); protein concentration was determined with the BCA protein assay reagent kit (as above). The fraction of the accumulated CDF that resides in bile canaliculi was quantified with the biliary excretion index, calculated using B-CLEAR technology (Qualyst, Inc., Research Triangle Park, NC) based on the following equation (Liu et al., 1999b): Biliary Excretion Index = $[\text{Accumulation}_{\text{cells+bile}} - \text{Accumulation}_{\text{cells}}]/\text{Accumulation}_{\text{cells+bile canaliculi}} \times 100\%$.

Lactate Dehydrogenase Activity Assay. Before the functional studies, cell culture medium was assayed for lactate dehydrogenase activity. Leakage of the intracellular enzyme into extracellular medium is inversely related to cell viability. Lactate dehydrogenase activity was assayed with kit 500C (Sigma Diagnostics, St. Louis, MO) using lactate dehydrogenase-catalyzed reduction of pyruvate to lactate, subsequent conjugation of the remaining pyruvate with 2,4-dinitrophenylhydrazine, and spectrophotometric detection (λ = 500 nm) of the pyruvate-2,4-dinitrophenylhydrazone conjugate at alkaline pH (Cabaud and Wroblewski, 1958).

Statistics. Data are reported as mean ± S.D., except where indicated otherwise. Statistical significance was evaluated with one-way or two-way ANOVA with Tukey's post hoc test. In all cases, $p < 0.05$ was deemed significant.

Results

Validation of siRNA Activity, Delivery, Efficacy, and Toxicity. siFL with proven knockdown effect was synthesized with the T7 RNA polymerase protocol and was used to examine the knockdown effect on the expression of firefly luciferase expressed from the pGL3-FL plasmid in HepG2 cells. HepG2 cells were treated with 0.2, 0.5, or 1.0 μg of siFL; 48 h after transfection, *R. reniformis* luciferase-normalized activity of firefly luciferase was decreased by 74, 89, and 94%, respectively (Fig. 1).

Cy3-labeled siRNA was used to examine siRNA delivery to hepatocytes before and after collagen overlay. siRNA was delivered successfully into hepatocytes before overlay (Fig. 2), but not after overlay (data not shown).

Preliminary studies evaluated the knockdown effects from three different transfection time points (3, 12, and 24 h); the best effect was observed at 12 h and 24 h after plating. In the present studies, all transfections were performed at 20 h after plating and the cells were subsequently overlaid at 24 h.

After 3 days in culture, the time at which transport protein levels and function were assessed, lactate dehydrogenase activity was not elevated in siMrp2- or siMrp3-transfected sandwich-cultured rat hepatocytes (94 ± 13% and 87 ± 3% of nontransfected control sandwich-cultured rat hepatocytes, respectively; mean ± S.E.M.).

Knockdown of Mrp2 Expression in Sandwich-Cultured Rat Hepatocytes. With the online program RNAi OligoRetriever, three candidate target sequences of Mrp2 cDNA (GenBank accession number NM_012833) were chosen, each 21 nucleotides long. These sequences targeted the 1157–1177, 1309–1329, and 2995–3015 nucleotides of the Mrp2 cDNA and were named siMrp2AB1, siMrp2AB2, and siMrp2AB3, respectively (see *Materials and Methods* for antisense sequences). Sandwich-cultured rat hepatocytes were treated with siMrp2AB1, siMrp2AB2, siMrp2AB3, and an equimolar mixture of the three (siMrp2ABmix). Different knockdown effects on Mrp2 were observed based on immunoblot analysis (Fig. 3, A and B). In four independent experiments, transfection with siFL showed no knockdown effect on Mrp2 or Mrp3. siMrp2AB1 exhibited the most potent effect, which resulted in a significant 51 ± 21% reduction in endogenous Mrp2 expression compared with sandwich-cultured rat hepatocytes transfected with siFL or nontransfected sandwich-cultured rat hepatocytes (Fig. 3B). siMrp2AB2 also produced a significant knockdown effect (40 ± 20%). siMrp2AB3 knocked down Mrp2 protein only

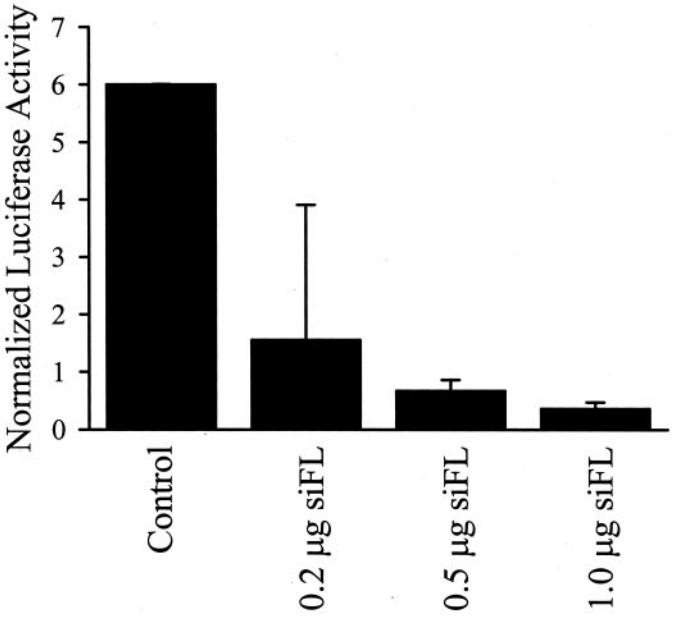

Fig. 1. Knockdown effect of siFL synthesized with the T7 RNA polymerase method. Firefly luciferase activity was examined in HepG2 cells transfected with pGL3-FL (firefly luciferase) and pRL-TK (*R. reniformis* luciferase) plasmids plus 0.2, 0.5, or 1.0 μg of siFL; activity was normalized against that of *R. reniformis* luciferase. Only pGL3-FL and pRL-TK plasmids were used in control transfection. Mean ± range (n = 2).

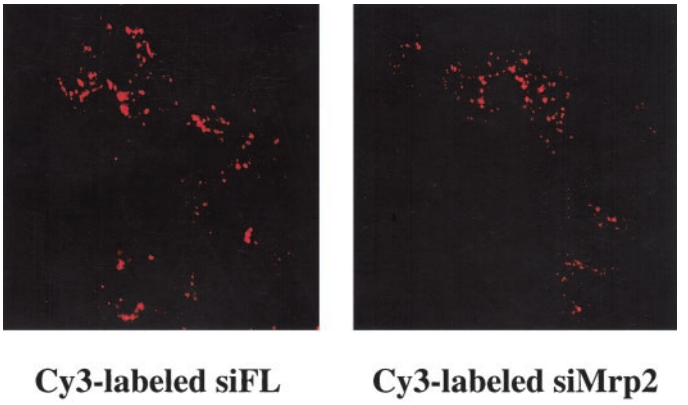

Fig. 2. Intracellular tracking of siRNA. siFL and siMrp2 were labeled with Cy3 reagent and transferred into hepatocytes before collagen overlay. Images were taken 24 h after transfection.

modestly (26 ± 18%). It is surprising that the equimolar mixture of these three sequences exhibited the least knockdown effect (~13% reduction).

Specificity of the siMrp2 Knockdown Effect. The specificity of the knockdown effect in siMrp2-treated cells was evident based on examination of the expression of Mrp3, an organic anion transport protein of ~45% homology to Mrp2. Immunoblot analysis indicated that the expression levels of Mrp3 in sandwich-cultured rat hepatocytes treated with siMrp2AB1, siMrp2AB2, siMrp2AB3, a mixture of these three, or siFL were the same as those in nontransfected cells (89 ± 11, 96 ± 15, 86 ± 11, 89 ± 19, and 96 ± 6% of nontransfected control, respectively; Fig. 3A).

Expression of radixin, a canalicular ezrin-radixin-moesin protein that plays a critical role in the correct localization of Mrp2 on the canalicular membrane, in sandwich-cultured rat hepatocytes increased over time in culture (day 2 = 111%, day 3 = 153%, and day 4 = 189% expression of day 1). Radixin expression levels were not altered in sandwich-cultured rat hepatocytes treated with siMrp2 or siFL compared with nontransfected sandwich-cultured rat hepatocytes (siMrp2AB1 = 95 ± 17%, siMrp2AB2 = 100 ± 8%, siMrp2AB3 = 103 ± 7%, siMrp2ABmix = 101 ± 8%, and siFL = 103 ± 10% of nontransfected control).

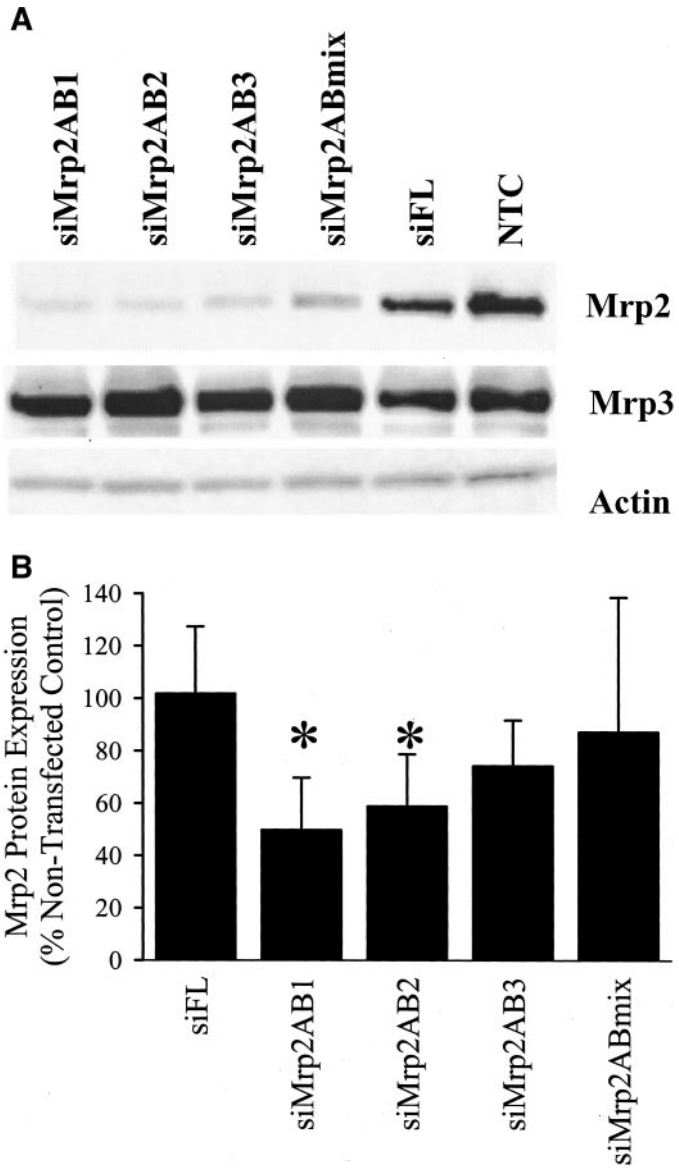

Fig. 3. Mrp2 and Mrp3 protein levels in siMrp2-treated sandwich-cultured rat hepatocytes. A, representative immunoblot of Mrp2 and Mrp3 expression in siMrp2-treated sandwich-cultured rat hepatocytes. NTC, nontransfected control. B, expression ratio of actin-normalized Mrp2 in siRNA-treated cells relative to nontransfected control. Mean ± S.D. (n = 4); *, $p < 0.05$ for siMrp2-transfected versus nontransfected control (one-way ANOVA with Tukey's post hoc test).

Mrp2 Function in siMrp2-Transfected Sandwich-Cultured Rat Hepatocytes. Consistent with decreased Mrp2 protein in siMrp2-transfected sandwich-cultured rat hepatocytes (Fig. 4A), fluorescence associated with the fluorescent Mrp2 substrate, CDF, in the bile canalicular networks of these hepatocytes was decreased markedly compared with sandwich-cultured rat hepatocytes treated with siFL or nontransfected sandwich-cultured rat hepatocytes (Fig. 4B). The biliary excretion index of CDF, measured immediately after incubation of sandwich-cultured rat hepatocytes with CDF diacetate, was ~45% lower in siMrp2-transfected sandwich-cultured rat hepatocytes relative to nontransfected controls (9.3 versus 16.5%; Fig. 4C). Accumulation of CDF in nontransfected cells + bile canaliculi was significantly higher than cellular accumulation (80.4 ± 3.9 versus 67 ± 5.5 pmol/mg of protein/10 min), indicating appreciable accumulation of CDF in bile canalicular networks. In contrast, in siMrp2-transfected sandwich-cultured rat hepatocytes, accumulation of CDF in cells + bile canaliculi was not significantly different from cellular accumulation of CDF (78.3 ± 6.3 versus 71.1 ± 7.8 pmol/mg of protein/10 min).

Modulation of Mrp3 Expression and Function in Sandwich-Cultured Rat Hepatocytes. Sandwich-cultured rat hepatocytes were treated with siMrp3 targeting the 1950–1970 nucleotides of the Mrp3 cDNA (GenBank accession number NM_080581; see *Materials and Methods* for antisense sequence). Immunoblot analysis showed a decrease of ~50% in Mrp3 protein expression, with no change in expression of Mrp2 compared with cells transfected with siFL and nontransfected cells (Fig. 4A). The functional assay with CDF, which also is an Mrp3 substrate, indicated that CDF fluorescence in bile canalicular networks was more intense than in siFL-transfected or nontransfected cells (Fig. 4B).

Immediately after incubation with CDF diacetate, no apparent effect of Mrp3 knockdown on CDF accumulation in cells + bile canaliculi or cells was noted (data not shown). The difference became apparent after a 30-min washout to allow CDF accumulated in hepatocytes to undergo appreciable excretion (Fig. 4D). CDF accumulation in siMrp3-transfected cells + bile canaliculi was significantly higher than in nontransfected cells + bile canaliculi (32.3 ± 2.5 versus 24.4 ± 4.3 pmol/mg of protein/10 min). In contrast, CDF cellular accumulation was not changed by Mrp3 knockdown (13.7 ± 2.2 versus 15.6 ± 4.0 pmol/mg of protein/10 min). The biliary excretion index of CDF after a 30-min washout was increased by ~60% (57.5% versus 36.2%) after Mrp3 knockdown.

Discussion

Although chemically synthesized siRNA is available commercially, the T7 RNA polymerase method proved to be a cost-effective way to synthesize small amounts of siRNA for preliminary screening (Donze and Picard, 2002). To ensure

the siRNA quality produced by this method, siFL with proven knockdown effect (Miyagishi and Taira, 2002) was synthesized and used to examine the knockdown effect on the expression of firefly luciferase expressed from the pGL3-FL plasmid in HepG2 cells. Firefly luciferase activity was decreased in a dose-dependent manner by siFL, indicating that siRNA synthesized by the T7 RNA polymerase method had a significant knockdown effect.

Unlike traditional monolayer culture of HepG2 cells, sandwich-cultured rat hepatocytes are cultured between two layers of gelled collagen. The top layer of collagen may limit the access of the siRNA/transfection reagent complex to hepatocytes. To ensure the efficient delivery of siRNA into sandwich-cultured rat hepatocytes, Cy3-labeled siRNA was used to examine the transfection efficiency before and after overlaying cells with collagen. siRNA was only delivered success-

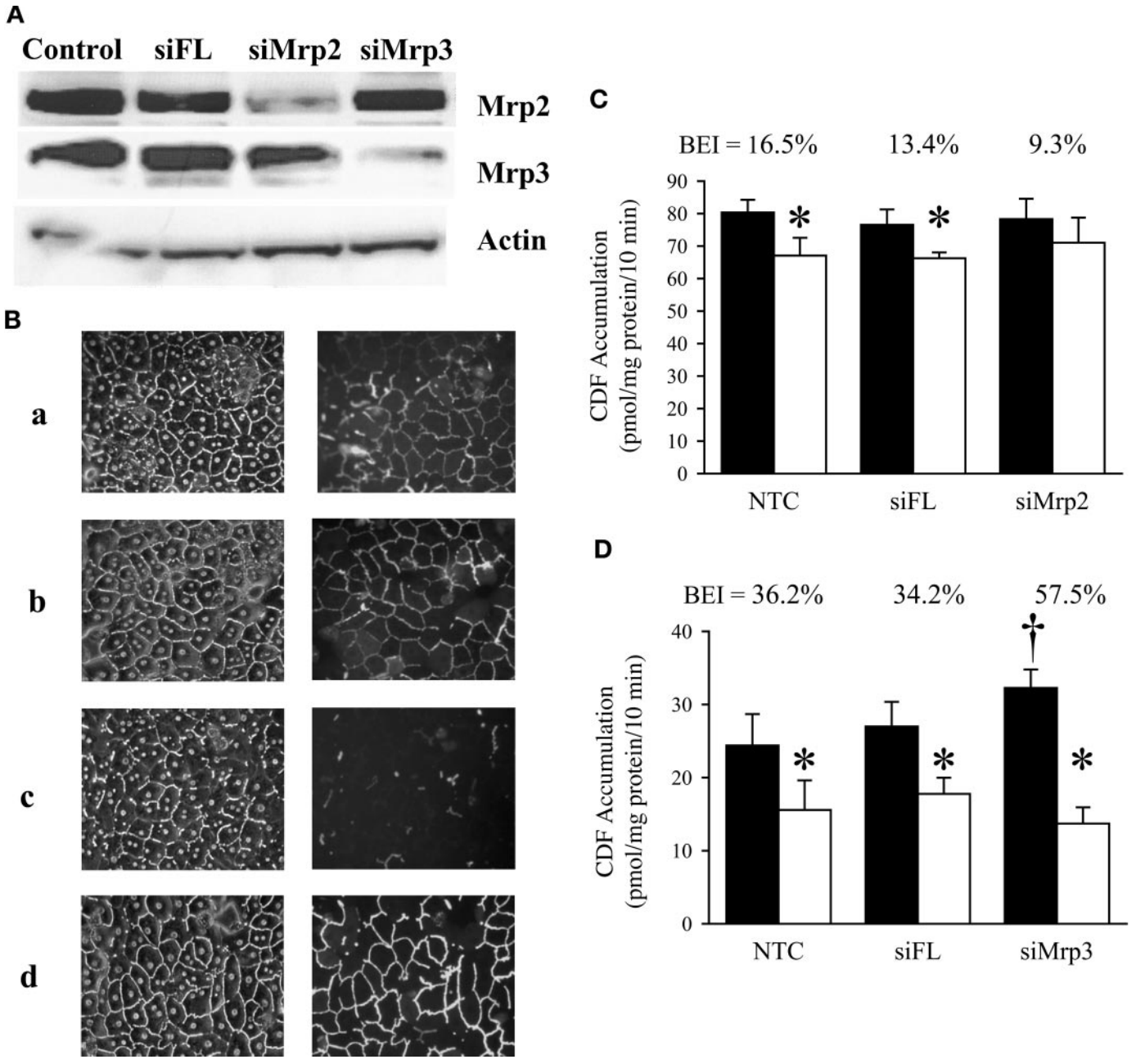

Fig. 4. Modulation of Mrp2 and Mrp3 expression and function in sandwich-cultured rat hepatocytes. A, representative immunoblot analysis of Mrp2 and Mrp3 expression in sandwich-cultured rat hepatocytes treated with siMrp2, siMrp3, siFL, and nontransfected cells. B, CDF fluorescence in the canalicular networks of sandwich-cultured rat hepatocytes treated with siRNA. Light microscopy image (left) and fluorescence microscopy image (right) are shown side by side in nontransfected cells (a), and in siFL-transfected (b), siMrp2-transfected (c), and siMrp3-transfected (d) cells after a 10-min incubation with 2 μM CDF diacetate and a washout period of ~10 min. C, CDF accumulation in nontransfected, siFL-transfected, and siMrp2-transfected sandwich-cultured rat hepatocytes in cells + bile canaliculi (closed bars) or cells (open bars) immediately after a 10-min incubation with 2 μM CDF diacetate. The biliary excretion index (BEI) of CDF is reported above the accumulation bars. D, CDF accumulation in cells + bile canaliculi (closed bars) or cells (open bars) in nontransfected, siFL-transfected, and siMrp3-transfected sandwich-cultured rat hepatocytes after a 10-min incubation with 2 μM CDF diacetate and a 30-min washout. The biliary excretion index (BEI) of CDF is reported above the individual accumulation bars. NTC, nontransfected control. Mean ± S.D. (n = 6); *, $p < 0.05$ for cells + bile canaliculi versus cells; †, $p < 0.05$ for siMrp2- or siMrp3-transfected versus nontransfected control (two-way ANOVA with Tukey's post hoc test).

fully into hepatocytes before overlay, indicating that the top layer of collagen limits access of siRNA to the cells.

Alterations in either the expression or localization of Mrp2 can lead to altered Mrp2 function. In vivo studies have demonstrated that radixin, the dominant bile canalicular ezrin-radixin-moesin protein in rodent liver, directly binds to the C-terminal cytoplasmic domain of Mrp2; the interaction between radixin and Mrp2 is necessary for the correct localization of Mrp2 on the canalicular membrane (Kocher et al., 1999; Kojima et al., 2003). When radixin was knocked out in mice, Mrp2 was mislocalized and malfunctioned (Kikuchi et al., 2002). After isolation of hepatocytes with collagenase, Mrp2 is internalized. In sandwich-cultured rat hepatocytes, Mrp2 relocalizes to the canalicular membrane over time in culture (Zhang et al., 2001). Expression of radixin in sandwich-cultured rat hepatocytes increased over time in culture, consistent with the relocalization of Mrp2 to the canalicular membrane. Radixin expression levels were not altered in sandwich-cultured rat hepatocytes treated with siMrp2 compared with those transfected with siFL or nontransfected sandwich-cultured rat hepatocytes. These data exclude the possibility that modulation of Mrp2 function by siMrp2 was mediated via the radixin pathway.

Treatment of sandwich-cultured rat hepatocytes with siMrp2 essentially produced a transient Mrp2-knockdown model (Figs. 3 and 4A). The decrease in Mrp2 function was demonstrated qualitatively by fluorescence microscopy using the fluorescent Mrp2 substrate, CDF. Fluorescence in the canalicular networks of sandwich-cultured rat hepatocytes treated with siMrp2 was markedly decreased compared with sandwich-cultured rat hepatocytes treated with siFL or nontransfected cells. From a quantitative standpoint, transfection of sandwich-cultured rat hepatocytes with siMrp2 decreased the biliary excretion index of CDF from 16.5 to 9.3%. This decrease (~45%) is in good agreement with the extent of Mrp2 protein knockdown (~50%). Decreased CDF excretion into the bile canaliculi was caused by decreased Mrp2 protein levels, and not compromised cell viability, as demonstrated by the lack of increase in lactate dehydrogenase activity in the medium of siMrp2-transfected sandwich-cultured rat hepatocytes.

Knockdown of Mrp3 did not change expression of Mrp2 compared with cells transfected with siFL or nontransfected cells (Fig. 4A). The functional assay with CDF, which also is an Mrp3 substrate, indicated that CDF fluorescence in the canalicular networks was more intense than in siFL-transfected or nontransfected cells (Fig. 4B). Transfection of sandwich-cultured rat hepatocytes with siMrp3 significantly increased accumulation of CDF in cells + bile canaliculi, but not in cells (Fig. 4D), resulting in an ~60% increase in the biliary excretion index of CDF. Knockdown of Mrp3 resulted in redirection of the route of CDF excretion into bile; hence canalicular fluorescence in siMrp3-treated sandwich-cultured rat hepatocytes was higher than in control cells. siMrp3-treated sandwich-cultured rat hepatocytes represent a transient Mrp3-knockdown model, which is unavailable in vivo.

As demonstrated by this work, RNA interference is a powerful tool for studying the role of specific transport proteins in disposition of substrates. The majority of current knowledge regarding the role of transport proteins in hepatobiliary drug disposition is derived from three types of experimental approaches: recombinant expression systems, knockout/mutant animals, and the use of "specific" inhibitors or inducers. Although much progress has been made with these tools in the study of transporter function, current experimental systems have limitations, which are discussed below. Many of these limitations are not relevant to RNA interference.

Recombinant protein may not always be representative of in vivo protein in the species of interest, because of differences in transcription, translation, and post-translational modifications in the host cell. For example, Sf9 cells, which are often used as the host system for expression of many ATP-binding cassette transporters, greatly underglycosylate the recombinant protein, resulting in transporters of lower molecular weight (Germann et al., 1990). Furthermore, recent research indicates that certain compounds may be substrates of more than one transporter at a given plasma membrane domain. For example, biliary excretion of estradiol-17β-glucuronide is mediated by both Mrp2 and P-glycoprotein (Takikawa et al., 1996). Likewise, hepatic uptake may be mediated by more than one transporter. The opioid peptide, [D-penicillamine^{2,5}]enkephalin, is taken up into hepatocytes by all three organic anion-transporting polypeptide isoforms present in the liver (Cattori et al., 2001). Therefore, accurate interpretation of the in vivo significance of recombinant protein transport activity may be very difficult.

Although knockout or naturally occurring mutant animals have advanced our understanding of the role of individual transporters in vivo, up-regulation of compensatory transport mechanisms in these animals may confound data interpretation. For example, in Mrp2-deficient Eisai hyperbilirubinemic rats, biliary excretion of taurocholic acid is impaired, not because Mrp2 mediates biliary excretion of this bile acid, but because basolateral Mrp3 is highly up-regulated in the livers of these rats (Akita et al., 2001). Mrp3 up-regulation results in increased basolateral excretion of taurocholate, limiting available substrate for biliary excretion. Interpretation of data obtained from knockout or mutant animals must take into consideration potential alterations in compensatory pathways, which may not always be well understood.

Inhibitors and inducers can be useful in studying the effect of known modulation of a transport pathway on drug disposition, but the effects of these modulators on other pathways must be considered in data interpretation. So-called "specific" inhibitors are often found to interact with multiple transport pathways as their disposition becomes more fully understood. For example, GF120918, which was initially thought to be a specific inhibitor of P-glycoprotein (P-gp, *Abcb1*), was shown recently to also inhibit the breast cancer resistance protein [Bcrp, *Abcg2* (Allen et al., 1999)]. Bromosulfophthalein, which traditionally has been used as a competitive inhibitor of basolateral organic anion-transporting polypeptides, is also a substrate for canalicular Mrp2 (Cattori et al., 2001; Cui et al., 2001). Inducers typically up-regulate protein by activation of transcription factors, which control the transcription of several genes, and thus may cause induction of more than one transporter and metabolic enzyme, again making data interpretation difficult (Wang and LeCluyse, 2003).

As demonstrated here, RNA interference allows specific and rapid knockdown of a protein of interest. Transfection of sandwich-cultured rat hepatocytes with siMrp2 specifically

decreased Mrp2 protein, but not the closely related Mrp3. Likewise, knockdown of Mrp3 did not affect Mrp2 protein levels. In naturally occurring mutants lacking Mrp2 (e.g., Eisai hyperbilirubinemic rats), Mrp3 is highly up-regulated in the liver (Akita et al., 2001). However, knockdown of Mrp2 did not alter Mrp3 protein levels 2 days after transfection of hepatocytes with siMrp2. The rate of protein knockdown with siRNA is dictated by the half-life of the protein, which for Mrp2 is apparently short enough that a significant decrease in Mrp2 levels can be achieved before a notable increase in Mrp3 occurs.

Overall, modulation of drug transporters by siRNA treatment in sandwich-cultured rat hepatocytes seems to be a feasible approach for studying the expression and function of drug transport proteins. RNA interference offers unique advantages over experimental designs used currently to study the role of transport proteins in drug disposition. RNA interference allows specific knockdown of a transport protein but maintains normal expression of other proteins relevant to the study of drug disposition, including compensatory transport mechanisms, in primary cells.

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Address correspondence to: Dr. Kim L. R. Brouwer, University of North Carolina School of Pharmacy, CB #7360, Room #2312 Kerr Hall, Chapel Hill, NC 27599-7360. E-mail: kbrouwer@unc.edu